Georgia State University iGEM Laboratory Manual

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Media

Purpose: Provide nutrition for microorganisms (bacteria/yeast) in liquid cultures

There is a wide variety of medias used in bacterial culture. LB or Lysogeny Broth is one of the most widely used medias in the culture of *E. coli*; however occasionally other more nutritionally rich broths may be used such as SOC media. Additionally, yeast has different nutritional needs and therefore different broths. Universally, however, the purpose of media is to provide a nutritionally rich environment that supports the growth and proliferation of bacterial cells. Since media is an extremely favorable growing environment for bacteria, it is essential that media be properly sterilized prior to use to protect from contamination from unwanted bacterial strains. Sterilization can be achieved by proper filtration or use of an autoclave. Once autoclaved, antibiotics are commonly added to the media to prevent unwanted growth and select for desired strains. It is important that antibiotics are added after autoclaving when the media has cooled to under 60°C because many antibiotics can be inactivated by heat. Although antibiotics will prevent some contamination, it is vital that proper aseptic technique is used when handling all media. A liquid culture is created by inoculating an aliquot of media with a bacterial colony.

LB Broth

Add the following to 800mL H_2O

- 10g Bacto-tryptone.
- 5g yeast extract.
- 10g NaCl.

Adjust pH to 7.5 with NaOH.
Adjust volume to 1L with dH_2O
Sterilize by autoclaving

Low Salt LB Broth

Add the following to 800 mL H_2O

- 10g Bacto-tryptone.
- 5g yeast extract.
- 5g NaCl.

Adjust pH to 7.5 with NaOH.
Adjust volume to 1L with dH_2O
Sterilize by autoclaving

SOC Media

Add the following to 800mL H_2O

- 20g Bacto Tryptone
- 5g Bacto Yeast Extract
- 2ml of 5M NaCl.
- 2.5ml of 1M KCl.
- 10ml of 1M $MgCl_2$
- 10ml of 1M $MgSO_4$
- 20ml of 1M glucose

Adjust volume to 1L with dH_2O
Sterilize by autoclaving

YPD

Add the following to 800mL H_2O

- 10g BactoYeast extract
- 20g BactoPeptone.
- 20g Dextrose.

Adjust volume to 1L with dH_2O
Sterilize by autoclaving

Agar plates are a solid growth medium for bacterial culture. Virtually all liquid medias can be combined with agar to create solidifying media that can be poured to create culture plates. As with regular liquid media antibiotics are usually added following sterilization. Culturing on a solid medium is useful because it allows for the isolation of individual colonies.

LB Agar

Add the following to 800ml H_2O

- 10g Bacto-tryptone
- 5g yeast extract
- 10g NaCl
- Adjust pH to 7.5 with NaOH
- Add 15g agar

Melt agar into solution on hot plate.
Adjust volume to 1L with dH_2O
Sterilize by autoclaving
Once cooled to touch, pour 20-25 mL of broth into each plate.

Preparation of Antibiotic Solutions

Purpose: inhibits unwanted growth and selects for desired construct

Antibiotics are used to limit contamination and select for desired constructs. Most iGEM parts are in one of three standard plasmids with three different antibiotic resistances: ampicillin, chloramphenicol, or kanamycin. iGEM designates antibiotic resistance of a plasmid through a one letter abbreviation in the backbone name: PSB#**X**# where **X** is A, C, or K.

*all antibiotics are made in a concentrated stock solution and when added to LB is added in a 1µL/mL ratio.

Antibiotic	Stock solution (mg/ml)	Solvent****	Storage temp (°C)	Working concentration		Function
				µg/ml	dilution	
Ampicillin*	50	H_2O	-20° C	50	1.0 µl/ml	Binds to the 50S ribosomal subunit and inhibits ribosomal peptide bond formation.
Chloramphenicol	35	EtOH or MetOH	-20° C	35	1.0 µl/ml	Inhibits protein synthesis.
Kanamycin	50	H_2O	+4°C- -20° C	50	1.0 µl/ml	Interacts with at least three ribosomal proteins, inhibiting protein synthesis and increasing translation errors.
Tetracycline***	15	EtOH, 70%EtOH	-20°C	15	1.0 µl/ml	Inhibits growth by preventing codon-anticodon interactions during translation.

* - Unstable solution - prepare just before use.

*** - Light sensitive, Mg^{2+} - inhibitor - do not use with minimal media.

Zeocin *Light Sensitive

Organism	Stock Solution (mg/mL)	Solvent	Storage Temp	Working Concentration		Function
				ug/mL	dilution	
E. coli	25-50	water, low salt LB	-20 -20	25-50	1 µl/ml 1 µl/ml	cleaves DNA
Yeast	50-300			50-300		

Preparing Competent E. Coli

Purpose: creates bacterial cells that readily uptake foreign DNA (DNA of interest)

Day 1

Streak the strain you wish to make competent onto an LB plate and incubate overnight 37°C.

Day 2

The next afternoon, pick a single colony into 10 mL of LB in a culture tube and *grow overnight* in a shaking incubator at 37°C.

At this point chill the following in the freezer:

- 50 mL falcon tubes
- 1L of sterile 10% glycerol
- 35 sterile cryovials (or microcentrifuge tubes), labelled with the strain name

Day 3

1. In the morning, inoculate 800 mL of LB in a flask with 1 mL of the overnight culture and grow at 37°C in a shaking incubator.
   NOTE: For optimal growth, ensure that volume of flask is filled with 70-80% air. Use this time to cool down the centrifuge
2. Grow the culture to an OD_{600} of 0.8 at 37°C. This should take around 2-3 hours.

FROM NOW ON KEEP EVERYTHING ON ICE AT ALL TIMES

3. Transfer 400mL of the culture to 8x pre-chilled 50 mL falcon tubes (or a suitably sized sterile centrifuge pot). Chill the tubes, and the remaining 400 mL on ice for 1 hour.
4. Centrifuge for 30 minutes at 3,900 rpm and 4°C then very carefully remove the supernatant.
5. Pour the remaining 400mL of culture into the tubes and repeat step 4.
6. Add 20mL of chilled 10% glycerol to each falcon tube and gently re-suspend the cells. Then make up the volume in each tube to 25 mL with 10% glycerol.
7. Centrifuge for 30 minutes at 3,900 rpm and 4°C then remove the supernatant.
8. **Repeat steps 6 and 7 twice**. On the final repeat, pool all of the cells into 1 Falcon tube, centrifuge as before then re-suspend in a final volume of 3mL in 10% glycerol.
9. Leave the cells on ice for 10 minutes then pipette 120μL into each cryovial.
10. Snap freeze the cells by placing the tube in a dry ice+EtOH bath. After snap-freezing, transfer immediately to the -80°C freezer.
    Save one sample (40μL) of competent cells to test transformation efficiency(pg10)

Polymerase Chain Reaction (PCR)

Purpose: Amplifies segment of DNA into millions of copies.

Thaw the PCR master mix. Vortex to mix and centrifuge briefly to collect all material in the bottom of the tube.

Mix together:

- 12.5 µl Master mix (Master Mix contains Taq polymerase, dNTPs, etc.)
- 1 µl Forward primer
- 1 µl Reverse primer
- 1 µl template DNA
- 4.5 µl sterilized water

Centrifuge briefly to collect in bottom of tube.

Put in thermocycler and select appropriate program.

*For Colony PCR, substitute "1 µl template DNA" with E. coli cells. Using a pipette tip, touch the bacterial colony from an agar plate that whose DNA you wish to amplify. Add a few bacterial cells to the PCR mix. Add an additional 1 µl of sterilized water (V_f=15.5µL) *Note: A huge quantity of cells is not needed for PCR to occur properly**

Fast Digestion of DNA using FastRestriction Enzymes

Purpose: Restriction enzymes cut DNA at specific sequences.

1. Mix together in the following order:
   - 14µL sterilized water
   - 2 µL 10X fast digest buffer
   - 2 µL DNA
   - 1 µL Each enzyme
2. Mix gently by pipetting and spin down.
3. Incubate at 37^0C for 5 min.*

*When using enzymes that are not Fast, incubate at 37^0C for 60 min.

Heat inactivation of Restriction Enzymes

Purpose: Stops restriction enzyme activity

Most restriction enzymes may be heat inactivated by heating to 65^0C for 20 min. Some enzymes require heating to 85^0C for 20 min. A list of these enzymes may be found on New England Biolab's website.

1% Agarose Gel (For Gel Electrophoresis)

Purpose: Visualize plasmid DNA for confirmation of DNA size

1. Mix 0.5g Agarose in 50 mL 1X TAE. *(1% Agarose Ratio 0.1g/10mL TAE)*
2. Heat in increments of 25 seconds until solution is clear in microwave. (Usually after it has boiled twice.) Swirling occasionally. (Total microwave time: ~60-70sec)
   Let cool until comfortable to touch.
3. Add 5µL GelRed. Swirl to mix. *(GelRed Ratio 1µL gel red:10mL agarose solution)*
4. Insert casting tray in electrophoresis machine so that the plastic sides are touching the walls and a tight seal is formed.
5. Pour liquid gel into casting tray. Add desired comb to form wells at one end of the gel.
6. Allow gel to solidify.
7. Reposition casting tray so that the wells are nearer the black *(negative)* electrode. Fill the machine to the fill line with 1X TAE. Remove comb.
8. Load 5µL Ladder to left-most well. Load samples into respective gel wells and run at 80V for 90min.

* Sample should be mixed with 6X loading dye before being loaded. For every 5µL sample add 1µL 6X loading dye, mix thoroughly. Be sure to always run a ladder with your sample.
DNA has an overall negative charge, therefore the agarose gel well should be near the black electrode. Upon voltage application the negatively charged DNA migrates to the red *(positive)* electrode. THINK--RUN TO RED

Gel Extraction of DNA (after running gel electrophoresis)

Purpose: extracts the DNA from the agarose gel for further analysis

Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

1. Weigh the gel slice in a colorless tube. Add 3 volumes Buffer QG to 1 volume gel (100 mg ~ 100 µl). For >2% agarose gels, add 6 volumes Buffer QG.

2. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved).Vortex the tube every 2–3 min to help dissolve gel.

3. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.

4. Add 1 gel volume of isopropanol to the sample and mix. Place a QIAquick spin column in a provided 2 ml collection tube.

5. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min. Discard flow-through and place the QIAquick column back into the same tube. For sample volumes of >800 µl, load and spin again.

6. If the DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, add 0.5 ml Buffer QG to the QIAquick column and centrifuge for 1 min. Discard flow-through and place the QIAquick column back into the same tube.

7. To wash, add 0.75 ml Buffer PE to QIAquick column and centrifuge for 1 minor. Discard flow-through and place the QIAquick column back into the same tube.
   *Note: If the DNA will be used for salt-sensitive applications (e.g., sequencing, blunt-ended ligation), let the column stand 2–5 min after addition of Buffer PE.

8. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min at 17,900 x g (13,000 rpm) to remove residual wash buffer.

9. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

10. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

Ethanol precipitation of DNA

Purpose: purifies and increases the concentration of the plasmid DNA extracted from Miniprep or gel extractions.

1. Determine the volume of your DNA solution.
2. Add 1/10 volume of 3M NaAc, pH 5.2 (If your original sample is 1 mL, you would add 100µL of NaAc).
3. Add 2.5 volumes of chilled 100% ethanol (If your original sample is 1 mL, you would add 2.5 mL of EtOH).
4. Incubate at -20°C for 20 minutes to allow precipitation.
5. Centrifuge for 15 min at 10,000 g or more at 4°C to pellet DNA.
6. Remove the supernatant, being careful not to dislodge the pellet.
7. Wash the pellet with chilled 70% ethanol, then centrifuge at 10,000 g or more for 15 min at 4°C.
8. Remove the supernatant and allow pellet to air dry.
9. Re-suspend the pellet in a small volume of TE or water.

If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Ligation of insert DNA into plasmid vector DNA

Purpose: attach 2 segments of plasmid together; generally a coding sequence, RBS, promoter or a combination of these to a plasmid backbone.

1. Thoroughly mix the 5X Rapid Ligation buffer prior to use.1.
2. Add the following to a microcentrifuge tube:
   - Linearized vector DNA (10-100 ng)
   - Insert DNA variable (at 3:1 molar excess over vector) (varies, typically less than 1µl)
   - 5X Rapid Ligation Buffer
   - 1 µl T4 DNA Ligase
   - Water, nuclease-free to 20 µl Total volume
3. Vortex and spin briefly to collect drops.
4. Incubate the mixture at 22°C for 5 min.
5. Use 2-5 µl of the ligation mixture for transformation. The reaction mixture can be stored at 0-4°C until used for transformation. Prior to electroporation, chloroform extract the ligation mixture and use 1 µl for the electroporation reaction.

Transformation of Competent E. coli by Electroporation

Purpose: Insertion of DNA of interest into E. coli. Amplifies entire DNA sequence.

1. Thaw the competent cells on ice. For each sample to be electroporated, place a 1.5ml microfuge tube and either a 0.1 or 0.2cm electroporation cuvette on ice.
2. In a cold, 1.5ml polypropylene microfuge tube, mix 40µl of the cell suspension with 1 to 2 µl of DNA (DNA should be in a low ionic strength buffer such as TE). Mix well and incubate on ice for ~1 minute.
3. Set the MicroPulser to "Ec1" when using the 0.1cm cuvettes. Set it to "Ec2" of "Ec3" when using the 0.2cm cuvettes. (*Note: The cuvettes we usually have are 0.2cm cuvettes.)*
4. Transfer the mixture of cells and DNA to a cold electroporation cuvette and tap the suspension to the bottom. Place the cuvette in the chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber. Pulse once.
5. Remove the cuvette from the chamber and **immediately** add 1ml of SOC medium to the cuvette. **Quickly** but gently re-suspend the cells with a Pasteur pipette.
6. Transfer the cell suspension to a 17x100mm polypropylene tube and incubate at 37°C for 1 hour, shaking at 225rpm.
7. Check and record the pulse parameters. The time constant should be close to 5milliseconds.
8. Plate on selective medium.

Transformation Efficiency

Purpose: Test to determine the competency level of the cells created; measures the number of transformed cells generated by a specific mass of supercoiled DNA.

Efficiency equation:

(#colonies on plate/ng DNA plated)*(1000ng/µg)

Miniprep of plasmid DNA

Purpose: extraction of plasmid DNA from bacterial cells.

1. Pick a single colony from a freshly streaked selective plate and inoculate a culture of 5 mL LB medium containing the appropriate selective antibiotic. Incubate for 12–16 h at 37°C with vigorous shaking (~200rpm).
   Growth for more than 16 h is not recommended since cells begin to lyse and plasmid yields may be reduced. Use a tube or flask with a volume of at least 4 times the volume of the culture.
   Harvest the bacterial cells by centrifugation at > 8000 rpm (6800 x g) in a conventional, table-top microcentrifuge for 3 min at room temperature (15–25°C).
2. Re-suspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a micro-centrifuge tube. Ensure that RNase A has been added to Buffer P1.
3. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be re-suspended completely by vortexing or pipetting up and down until no cell clumps remain
4. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA.
5. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.
6. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube4–6 times.
7. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting upto 10 times. The solution should become cloudy.If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.
8. Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.A compact white pellet will form.
9. Apply the supernatants from step 4 to the QIAprep spin column by decanting orpipetting.
10. Centrifuge for 30–60 s. Discard the flow-through.
    Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through
    This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step
11. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.

12. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.

    Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

13. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprepspin column, let stand for 1 min, and centrifuge for 1 min.